Are the GCF levels of MCP-1 and RANTES elevated in Peri-partum women with periodontitis as compared to healthy controls?

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ABSTRACT

The study objective was to compare levels of gingival crevicular fluid (GCF), MCP-1 and RANTES in peri-partum women with periodontal disease to healthy controls. This is secondary analysis of Oral Conditions and Pregnancy (OCAP), a case-control study. Subjects (N=80) were stratified post-hoc based on periodontal status. An oral exam was performed within 72 hours after delivery. Cases (n=42) had moderate to severe/periodontitis ($\geq$15 sites with PD$\geq$ 4mm). Controls (n=38) had a healthy periodontium (PD$\leq$ 3mm). Pregnancy history was obtained through charts and interviews. Statistical tests included chi-square and *t*-test. Groups were balanced for age (p=0.57) but not race. Cases and controls were 17% and 82% Caucasian, respectively (p<0 .0001). There were no statistically significant (SS) differences between cases and controls. MCP-1 and RANTES were lower in cases than controls although not SS. There is no significant difference between MCP-1 and RANTES levels in women with periodontitis compared with healthy controls.

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ABBREVIATIONS

Actinobacillus actinomycetemcomitans	*Aa*
African American	AA
Chemotactic Cytokines	CC
Gingival Crevicular Fluid	GCF
Human immunodeficiency virus	HIV
Acquired immunodeficiency virus	AIDS
Interleukin 1 alpha	IL-1α
Interleukin 1 beta	IL-1β
Interleukin-4	IL-4
Interleukin-6	IL-6
Interleukin-8	IL-8
Interleukin-10	IL-10
Interleukin-12	IL-12
Interleukin-16	IL-16
Junctional Epithelium	JE
Lipopolysaccharide	LPS
Low birth weight	LBW
Macrophage Inflammatory Protein- 1alpha	MIP-1α
Macrophage Inflammatory Protein-2	MIP-2
Matrix Metalloproteinase - 1	MMP-1
Matrix Metalloproteinase – 8	MMP-8
Monocyte Chemoattractant Protien-1	MCP-1
Monocyte Chemoattractant Protien-5	MCP-5

National Health and Nutrition Examination Survey III	NHANES III
Neonatal Intensive Care Unit	NICU
Oral Conditions and Pregnancy	OCAP
Periodontal Disease	PD
Polymorphonuclear leukocytes	PMNs
Preterm delivery	PTD
Prostaglandin-2	PGE_2
Tumor Necrosis Factor – alpha	$TNF_{-\alpha}$
United States of America	US

INTRODUCTION

Periodontal diseases (PD) include a group of chronic inflammatory diseases that affect the supporting structures of the teeth involving complex inflammatory interactions with the host, leading to potential tooth loss. [1,2] As one of the most common causes of tooth loss, and the most prevalent form of bone pathology, PD are a significant modifying factor of an individual's system.[1, 3] Periodontitis has been associated with cardiovascular diseases[4, 5], diabetes[6], neurological diseases[7, 8], certain types of cancers[9, 10], and rheumatoid arthritis.[11, 12] Further research has shown that chronic oral infections are also negatively associated with pregnancy, more specifically preterm and low birth weight deliveries.[13-21] Preterm delivery (PTD) is the leading cause of neonatal mortality in the US initiating a movement toward a deeper exploration of the potential association between PD and adverse pregnancy outcomes.[22]

There is an increasing body of evidence concerning the progression of periodontitis, suggesting that cytokines and chemokines, inflammatory mediators involved with the chemotaxis of leukocytes, may also contribute to a systemic promotion of PTD and LBW.[23-25] Due to complexity of the immune system, the response occurring due to the increasing bacterial challenge of PD makes it difficult to determine exactly how cytokines and chemokines are involved.[26] However, the proposed cytokine and chemokine relationship to disease progression make their connection to pregnancy an interesting target for further investigation.

Monocyte chemoattractant protein-1 (MCP-1) and Regulated upon activation, t-cell expressed or secreted (RANTES) were chosen for this study due to their potent chemotaxis of specific leukocytes involved in inflammatory progression. Although neither of these inflammatory mediators have been definitely associated with pregnancy outcome, they are Involved with several gestational events. The endometrium, the epithelial tissue that forms the

lining of the uterus, has been shown to produce MCP-1 and RANTES, along with many other inflammatory mediators.[27] Current research also reports that MCP-1 is present in amniotic fluid at all periods of gestation, as evidenced by a cross-sectional study conducted in 2005 by Esplin *et al*.[23] The purpose of this secondary analysis was to compare GCF levels of MCP-1 and RANTES in peri-partum women with periodontitis to healthy controls.

REVIEW OF THE LITERATURE

Periodontal Diseases

Periodontal diseases include a group of chronic inflammatory diseases that affect the supporting tissues of the teeth and encompass negative activities such as destruction of periodontal tissues that lead to potential tooth loss.[1-2, 28-29] Microorganisms and microbial products in dental biofilm are the mechanisms by which the initiation of the inflammatory reaction occurs, resulting in the destruction of the periodontium.

All PD begins as an acute phase, also known as gingivitis. However, not all gingivitis progresses into PD. The host initiates the immune response to the acute infection with a cascade of cytokines and prostaglandins, released from the JE cells into the nearby blood vessels.[30-32] The presence of the cytokine PGE_2 stimulates dilation of blood vessels, providing a means for the translocation of PMNs into the base of the JE.[32] Once in this region, this immune response is primarily dominated by a specific balance of PMN's that engulf the periodontopathogens, originally aiding in the healing process.[1, 33] If the host is unable to eliminate the microorganisms, the microbial composition of the acute gingivitis becomes more pathogenic and migrates through the tissues of the JE and into the connective tissues. [34-37] The original release of cytokines and prostaglandins continues, increasing in amount and intensity in response to higher concentrations of periodontopathogens.[1] If this process is not arrested, the virulence of the pathogens overcomes the host's ability to eliminate the repair the destruction, leading to a chronic phase of periodontitis, mobility and tooth loss.[32]

During the chronic phase of periodontitis, the infection has created periodontal pocketing, where gram-negative bacteria, most commonly *P. gingivalis, A. a, T. forsythias,* and *Treponema*

denticola, are responsible for the continued production of inflammatory cytokines, such as IL-1β, IL-6, IL-8 PGE_2 and TNFα.[37] In a prolonged exposure to inflammation, the production of endotoxin, also known as lipopolysaccharide (LPS) initiates the release of proinflammatory cytokines such as MIP-1a, MIP-2, MCP-5 and IL-8. [37] At this point the host response that has occurred initiates both direct and indirect destruction. [37] The bacterial by-products and endotoxins cause direct oral tissue damage by breaking down the periodontium and supporting structures. As these by-products and endotoxins migrate sub-gingivally and become more pathogenic, bleeding on probing evidences the indirect damages, caused by the cytokines and bacteria as they begin systemic circulation.[32]

The Oral Systemic Interface

Investigators have suggested that chronic oral infections could have a significantly negative relationship to systemic health issues, based on the ability of PD to modify normal biological functions by influencing the severity of many chronic health conditions.[3] Cardiovascular diseases, diabetes, neurological diseases, certain types of cancers and rheumatoid arthritis, have all been associated with periodontitis.[4-12] Further research has shown that chronic oral infections are also negatively associated with pregnancy, more specifically preterm and low birth weight deliveries.[13-21]

In 2001, Jeffcoat and colleagues reported that nearly 50% of the US population suffers from some form of PD whether it is gingivitis or periodontitis.[38] When assessing the oral health status of women in the US, studies show that PD affects 23 % between 30-54 years of age. [39, 3]

Cytokines and chemokines have been associated with PD, as the possible mechanism by which tissue destruction occurs, due to their potent chemoattraction of leukocytes. [40-41] Although research concerning oral disease and systemic illness has been ongoing and is showing associations in some areas, additional research is needed to determine the mechanisms by which these associations exist.

Role of Cytokines and Chemokines in Inflammatory Processes

Cytokines are low molecular weight secreted proteins, aiding in host in regulation of the response by sending phagocytic cells to sites of inflammation. [26, 40, 42 -44] Due to this function, current research states they are possibly responsible for regulating the immune system by influencing the duration and intensity of its inflammatory response. [40-41, 44-45] The immune system has many cell populations that produce cytokines, primarily any nucleated cell, such as fibroblasts, endothelial cells, PMNs and epithelial cells. [26, 32, 46] Rarely do cytokines function independently, as they are pleiotrophic and frequently defined as a network. Cytokine interaction begins as a single cytokine initiates the action of many others, providing for stimulation of many cell surface receptors, which finally leads to negative or positive influences of cytokines on various cell processes. [30, 41]

Before cytokines were well understood, they were referred to in relation to the cells in which they most significantly influenced. [46] For example, lymphokines (produced by lymphocytes), monokines (produced by monocytes), chemokines (chemotactic cytokines) and interleukins (cytokines made by one leukocyte but act on other leukocytes) were various ways to describe these inflammatory mediators. [46-47] Due to these various functions and properties, cytokines are intricately involved with proliferation, development, differentiation, homeostasis, regeneration, repair and inflammation, with their influence significantly dependent upon cell type and time. [30, 44]

In 1992 the term “chemokine” was adopted to incorporate comparable “chemotactic cytokines” responsible for attraction of various leukocytes such as neutrophils, monocytes or lymphocytes to sites of infection. [48] They are secondary pro-inflammatory mediators, most commonly stimulated by the presences of the cytokines IL-1 or TNFα. [49] A variety of cells within the periodontal tissues are responsible for the production of chemokines such as fibroblasts, endothelial cells, macrophages, osteoclasts, epithelial cells, monocytes, lymphocytes, PMNs and mast cells. [35-36, 50, 49] Chemokines are divided into 2 subgroups, based on their

homeostatic and inflammatory functions.[1] Their homeostatic functions are concerned with the migration of cells during normal tissue maintenance and development, whereas the inflammatory chemokines are stimulated by pathogens, and most commonly by cytokines.[1] During the last 10-12 years, research has identified nearly 40 –50 total chemokines, which are divided into four structure-based subfamilies.[51]

During the process of PD, it is important to study the balance of cytokines, chemokines and other inflammatory mediators released from destructed tissues, as these events are potentially related to the progression of the disease. [1, 30, 41, 52] Inflammatory cytokines are secreted in the GCF and blood stream during the sequence of the early host response to acute oral infection.[45] The most common inflammatory cytokines are IL-1α, IL-1β, IL-6, IL-8 and TNFα and are primarily responsible for disease initiation and duration.[30, 53] If the host response to oral infection is only temporary, the inflammation serves as immune protection and begins the wound healing.[41] This is marked by the anti-inflammatory cytokines, such as IL-10, IL-4, and IL-12 primarily responsible for the reduction of inflammatory reactions.[26, 44] However, after prolonged exposure to periodontopathogens, the cytokines released by PMNs, such as IL-1a, IL-1b, TNF-a, IL-6 and IL-8 are responsible for initiating the secondary pro-inflammatory response, which consists of chemokines as well.[37] During this phase of inflammation, considerable tissue damage can potentially occur, leading the host into chronic inflammation and further destruction.[1, 26]

Chemokine research suggests their relation to the severity of PD is based upon their role in the recruitment of leukocytes, white blood cells that significantly influences the immune response.[54-57] To compliment this theory, chemokines have also been documented in the activation of osteoclasts, which promotes bone resorption, representing the destructive nature of disease progression.[1, 58]

There is an increasing body of evidence, concerning the progression of periodontitis, suggesting that cytokines and chemokines may also contribute to a systemic promotion of PTD and LBW specifically by means of their involvement with leukocytes and inflammation.[24-25, 59]

Even in the absence of inflammation, leukocytes are associated with the rebuilding of the endometrium when implantation does not occur, cervical preparation for labor as well as delivery.[57, 60-61] Leukocytes have also been documented as predominate producers of chemokines.[59] Chemokines may also be involved with the process of cell differentiation of the uterine tissues in preparation for pregnancy shortly after implantation.[59]

Due to complexity of the immune system, the response occurring due to the increasing bacterial challenge of PD and other inflammatory processes makes it difficult to determine exactly how cytokines and chemokines are involved with pregnancy outcomes.[26] However, because about 25% of all preterm delivery (PTD) and low birth weight (LBW) cases in the US still remain unexplained research is initiating a movement toward a deeper exploration of this potential association.[17, 19]

An association between periodontitis and pregnancy outcomes has been established and well documented, but unfortunately the pathogenesis remains unclear.[19-20, 38, 62-65] The current hypothesis concerning the relationship between pregnancy, the immune system and response to active periodontal infection is based on the coupling of several inflammatory mediators involved with both the progression of PD, labor and delivery.[19-20, 66-67] Many of the complex immune responses, primarily influenced by multifaceted cytokine and chemokine activity, occurring due to the presence of oral gram-negative microbes of PD are also occurring during normal pregnancy, labor and delivery.[25, 37, 42, 59, 67-68]

On several occasions, the literature has reported that PGE_2, TNFα IL-1, IL-6 and IL-8 are normal inflammatory mediators involved with the body's progressive preparation for labor.[37, 67, 69] During normal pregnancy, levels of these of prostaglandins and cytokines, along with increasing progesterone and estrogen from the placenta, contribute to significant hormonal changes as they continually increase.[68] They continue to increase until a critical threshold is reached in the amniotic fluid, encouraging uterine contractions, cervical dilation and delivery of the baby.[37] As previously mentioned many of these same prostaglandins and cytokines, such as PGE_2, TNFα,

IL-Iβ, IL-6, and IL-8, are produced in response to the presence of gram-negative bacteria involved with PD.[37, 67] In the case that a pregnant woman is experiencing PD, high levels of prostaglandins and cytokines could possibly begin systemic circulation.[37, 70] This encourages the amniotic levels of the mediators to peak prematurely and the untimely release of the cytokine PGE_2 decreases the normal exchange of blood and nutrients to the fetus, thus significantly increasing the risk for PTD and/or LBW.[1, 62]

Adverse Pregnancy Outcomes: Impact on Healthcare

Attention to the concern that periodontitis is significantly related to pregnancy was initiated in the mid 1990's when Offenbacher and colleagues conducted a landmark case control study of 124 pregnant and postpartum mothers. After controlling for known risk factors for PTD and LBW, the study concluded that there was a trend toward women who experienced PTD and pPROM to have more severe periodontitis than mothers of full-term normal birth weight infants.[19] The proposed association was based on the hormonal changes associated with pregnancy that are significantly associated with other immune system cells that respond to chronic inflammation. Additionally, in 1998 Dasanayake *et al*. conducted a case-control study of 110 women and concluded that the presence of healthy gingiva, determined a lower risk for LBW infants.[16]

Current findings strengthen this theory that hormonal changes, primarily concerning estrogen and progesterone, are fully initiated around the 2nd month of pregnancy.[37, 66, 71] This could potentially be attributable to the 60-75% of pregnant women who begin to display clinical signs of gingivitis, making this the most common oral disease during pregnancy.[1, 37] The presence of gingivitis, even in an acute phase, increases a woman's risk for developing PD during and/or up to several months after pregnancy.[66, 71]

Since the landmark studies conducted in the 1990's, medical research has discovered that PTD is the leading cause of neonatal mortality in the US.[17] It is also responsible for 50% of the neurological handicaps occurring to children born too early.[72] Based on race and ethnicity, the

rate of PTD is highest for black infants (18.1%) followed by Native Americans (13.8%), Hispanics (12%), Caucasian (11.5%) and Asians (10.5%). Maternal age is also significantly related to a mother's chances of experiencing a PTD. During a period between 2003-2005, PTD was highest for women 40 years and older (16.6%), followed by women under age 20 who experienced 14.5% of all PTD.[17]

Not only has the management of an increasing number of PTD and LBW infants that survive in the NICU placed a significant monetary burden on the US health system, but PTD and LBW babies face multiple serious health issues all throughout life, which also demand monetary support. In 2005, the March of Dimes reported that the US spent about $26.2 billion dollars annually or $51,600 for every PTD infant, who represent about 10 % of all live North American births.[17] The average first-year medical costs, including both inpatient and outpatient care, were about 10 times greater for preterm infants ($32,325) than for term infants ($3,325).[17] It can also be reported that 60 **%** of all neonatal mortality is attributable to PTD which also accounts for nearly 50 **%** of all perinatal health care costs in the US. [38] Currently, the March of Dimes is leading a national campaign, whose goal is to meet the Healthy People 2010 objective of reducing the number of premature births to no more than 7.6 **%**, nearly half of the 12.1 **%** of premature births in 2002.[73]

With these statistics in mind, it is important to remember that a woman's pregnancy experience with PD influences her oral health status, and more importantly, it significantly increases the risk of compromising the health of her fetus.[74]

Role of MCP-1 and RANTES in Pregnancy Outcomes

Because inflammation is hallmark of several female reproductive processes and due to MCP-1 and RANTES involvement with many other inflammatory processes, this makes these two chemokines an interesting target for further research in association to pregnancy outcomes. MCP-1 is involved with several aspects of uterine function, primarily present during the pre-menstrual stages and shortly after implantation of the fetus on the placental wall.[59] Furthermore,

the endometrium, epithelial tissue that forms the lining of the uterus, has been shown to produce MCP-1 and RANTES among other chemokines such as IL-8, and MIP-1α.[27] RANTES also plays an important role in recruiting t-lymphocytes, monocytes, basophils and eosinophils into the endometrium.[59]

MCP-1 is a significant chemoattractant for specific subsets of lymphocytes, such as monocytes and macrophages.[32, 42, 54, 75] As the best-characterized member of the CC family, MCP-1 is expressed by monocytes, endothelial cells, fibroblasts and T-cells, primarily on the basal layer of epithelial tissues. [43, 49, 54, 76] MCP-1 has been associated with many inflammatory conditions such as granulamatous disease rheumatoid arthritis, heart disease, bone trauma and asthma. [54, 77] MCP-1 is related to the stages of oral infection by means of its monocyte chemotactic ability, as it has been known to increase with increasing inflammation. [45, 53]

RANTES is a member of the CC family as well, located on chromosome #17 in humans. [1] RANTES is believed to be a chemotactic factor for monocytes, macrophages, eosinophils, basophils and t-lymphocytes explaining why it's activity is commonly studied in conjunction with MCP-1.[40, 42, 54, 76, 78-79] Although research has not determined if it is directly related to chronic PD, Gamonal *et al.* conducted two studies in 2000, reporting that RANTES, along with Interleukin-10 (IL-10) were the only two chemokines exclusively associated with periodontitis patients. [24, 42] A study conducted by Nelson *et al.* also reported that events occurring in relation to the RANTES receptors indicate that RANTES is involved with acute and chronic stages of PD.[80]

During the studies of RANTES and PD, although still controversial, it was discovered that RANTES is potentially a significant chemoattractant of Th1 cells. [49, 81] Because Th1 cells are the cell-mediated response, primarily intensifying the presence of the immune cells that invade inflammatory lesions, this supports the evidence that RANTES could be related to PD progression as well. [32]

For decades, research has consistently reported that an abundance of macrophage and monocyte cells are present in active sites of PD. [51, 55, 82-85] Macrophages constitute about 5-30% of the infiltrating cells in inflamed periodontal lesions and are often the most abundant cell of the chronic oral infection. [30, 43] Because many cytokines and chemokines have been associated with the recruitment of these cells to sites of inflammation, this suggests that these mediators are significantly involved with the immune response to periodontitis.[55] In 1993, Hanazawa *et al.* suggested that MCP-1 plays a role in the migration of monocytes into the periodontal tissues by reported the presence of MCP-1 in periodontitis patients and it's absence in periodontally healthy patients.[43] However, this study did not distinguish if MCP-1 levels increased with the severity of PD or if they were only associated with the presence of inflammation. Gamonal *et al* in 2000 also reported MCP-1's chemotactic ability on macrophages, is possibly causing the increase in severity of PD, due to an increased number of macrophages found in the GCF of active sites of PD. [24] Furthermore, they conducted the 1st study to show a presence of RANTES levels in patients with periodontitis and absence of RANTES in healthy patients. [42]

Research has shown several normal physiologic functions of female reproduction are initiated and regulated by inflammatory processes. [59] However, just as the literature concerning MCP-1, RANTES and PD is conflicting, the literature concerning these chemokines and pregnancy outcomes is inconsistent as well. In 1999, Athyde *et al.* concluded that women with preterm labor who also delivered preterm had higher amniotic fluid concentrations of RANTES than those who delivered term.[25] Microbial invasion of the amniotic cavity was also associated with a significant increase in median amniotic fluid RANTES in both preterm and term labor.[25] Additionally in 2003 Jacobsson *et al.* concluded that MCP-1 levels were higher in women experiencing preterm labor in association with inflammation than in non-laboring women, suggesting that MCP-1 is only elevated in the presence of inflammation, pPROM and /or the microbial invasion of the amniotic cavity.[86]

In contrast to these findings, a cross-sectional study conducted by Esplin *et al.* in 2005 reported that amniotic levels of MCP-1 are increased in relation to all pathways of delivery, regardless of the presence or absence of inflammation. [23] Also in 2005, Tornblom *el al*. reported that MCP-1 levels were significantly increased during non-infected labor as compared with non-laboring women, again suggesting that MCP-1 is elevated in response to the gestational event and not necessarily the inflammation.[87] Finally, Ramhorst *el al.* concluded in 2007 that failure of RANTES levels to increase might be associated with increased risk for miscarriage while an epidemiological study, also conducted in 2007 found that elevated concentrations of RANTES, among other chemokines, were associated with higher risk of miscarriage.[88-89]

The vital question research asks today is whether or not cytokines and chemokines are responsible for any of the progressive pathogenesis, associated with inflammatory diseases.[51] Because of the current limitations in the literature concerning the association between PD, MCP-1, RANTES and pregnancy, the purpose of this secondary analysis was to compare the GCF levels of MCP-1 and RANTES in peri-partum women with periodontitis to healthy controls and correlate these levels with clinical parameters. In conjunction with evidence-based proof about the oral systemic link, studying these two chemokines prefaces a movement to accelerate the enhancement of oral and general health for all women and their children.

INTRODUCTION AND REVIEW OF THE LITERATURE

Periodontal diseases (PD) include a group of chronic inflammatory diseases that affect the supporting structures of the teeth involving complex inflammatory interactions with the host, leading to potential tooth loss.[1-2, 28-29] As one of the most common causes of tooth loss, and the most prevalent form of bone pathology, PD are a significant modifying factor of an individual's system.[1, 3] Periodontitis has been associated with cardiovascular diseases, diabetes, neurological diseases, certain types of cancers, and rheumatoid arthritis.[4-12] Further research has shown that chronic oral infections are also negatively associated with pregnancy, more specifically preterm and low birth weight deliveries.[13-21] Preterm delivery (PTD) is the leading cause of neonatal mortality in the US initiating a movement toward a deeper exploration of the potential association between PD and adverse pregnancy outcomes.[22]

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All PD begins as an acute phase, also known as gingivitis. However, not all gingivitis progresses into PD. The host initiates the immune response to the acute infection with a cascade of cytokines and prostaglandins, released from the JE cells into the nearby blood vessels.[30-32] The presence of the cytokine PGE_2 stimulates dilation of blood vessels, providing a means for the translocation of PMNs into the base of the JE.[32] Once in this region, this immune response is primarily dominated by a specific balance of PMN's that engulf the periodontopathogens, originally aiding in the healing process.[33, 1] If the host is unable to eliminate the microorganisms, the microbial composition of the acute gingivitis becomes more pathogenic and migrates through the tissues of the JE and into the connective tissues. [34-37] The original release of cytokines and prostaglandins continues, increasing in amount and intensity in response to higher concentrations of periodontopathogens.[1] If this process is not arrested, the virulence of the pathogens overcomes the host's ability to eliminate the repair the destruction, leading to a chronic phase of periodontitis, mobility and tooth loss.[32]

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During the process of PD, it is important to study the balance of cytokines, chemokines and other inflammatory mediators released from destructed tissues, as these events are potentially related to the progression of the disease.[1, 30, 41, 52] Inflammatory cytokines are secreted in the GCF and blood stream during the sequence of the early host response to acute oral infection. [45] The most common inflammatory cytokines are IL-1α, IL-1β, IL-6, IL-8 and TNFα and are primarily responsible for disease initiation and duration.[30, 53] If the host response to oral infection is only temporary, the inflammation serves as immune protection and begins the wound healing.[41] This is marked by the anti-inflammatory cytokines, such as IL-10, IL-4, and IL-12 primarily responsible for the reduction of inflammatory reactions.[26, 44] However, after prolonged exposure to periodontopathogens, the cytokines released by PMNs, such as IL-1a, IL-1b, TNF-a, IL-6 and IL-8 are responsible for initiating the secondary pro-inflammatory response, which consists of chemokines as well.[37] During this phase of inflammation, considerable tissue damage can potentially occur, leading the host into chronic inflammation and further destruction.[1, 26]

Chemokine research suggests their relation to the severity of PD is based upon their role in the recruitment of leukocytes, white blood cells that significantly influences the immune response.[54-57] To compliment this theory, chemokines have also been documented in the activation of osteoclasts, which promotes bone resorption, representing the destructive nature of disease progression.[1, 58]

There is an increasing body of evidence, concerning the progression of periodontitis, suggesting that cytokines and chemokines may also contribute to a systemic promotion of PTD and LBW specifically by means of their involvement with leukocytes and inflammation.[24-25, 59] Even in the absence of inflammation, leukocytes are associated with the rebuilding of the endometrium when implantation does not occur, cervical preparation for labor as well as delivery.[57, 60-61] Leukocytes have also been documented as predominate producers of

chemokines.[59] Chemokines may also be involved with the process of cell differentiation of the uterine tissues in preparation for pregnancy shortly after implantation.[59]

Due to complexity of the immune system, the response occurring due to the increasing bacterial challenge of PD and other inflammatory processes makes it difficult to determine exactly how cytokines and chemokines are involved with pregnancy outcomes.[26] However, because about 25% of all preterm delivery (PTD) and low birth weight (LBW) cases in the US still remain unexplained research is initiating a movement toward a deeper exploration of this potential association. [17, 19]

An association between periodontitis and pregnancy outcomes has been established and well documented, but unfortunately the pathogenesis remains unclear. [19-20, 38, 62-65] The current hypothesis concerning the relationship between pregnancy, the immune system and response to active periodontal infection is based on the coupling of several inflammatory mediators involved with both the progression of PD, labor and delivery. [19-20, 66-67] Many of the complex immune responses, primarily influenced by multifaceted cytokine and chemokine activity, occurring due to the presence of oral gram-negative microbes of PD are also occurring during normal pregnancy, labor and delivery.[25, 37, 42, 59, 67-68]

On several occasions, the literature has reported that PGE_2, TNFα IL-1, IL-6 and IL-8 are normal inflammatory mediators involved with the body's progressive preparation for labor.[37, 67, 69] During normal pregnancy, levels of these of prostaglandins and cytokines, along with increasing progesterone and estrogen from the placenta, contribute to significant hormonal changes as they continually increase.[68] They continue to increase until a critical threshold is reached in the amniotic fluid, encouraging uterine contractions, cervical dilation and delivery of the baby.[37] As previously mentioned many of these same prostaglandins and cytokines, such as PGE_2, TNFα, IL-Iβ, IL-6, and IL-8, are produced in response to the presence of gram-negative bacteria involved with PD.[37, 67] In the case that a pregnant woman is experiencing PD, high levels of

prostaglandins and cytokines could possibly begin systemic circulation. [37, 70] This encourages the amniotic levels of the mediators to peak prematurely and the untimely release of the cytokine PGE_2 decreases the normal exchange of blood and nutrients to the fetus, thus significantly increasing the risk for PTD and/or LBW.[1, 62]

Attention to the concern that periodontitis is significantly related to pregnancy was initiated in the mid 1990's when Offenbacher and colleagues conducted a landmark case control study of 124 pregnant and postpartum mothers. After controlling for known risk factors for PTD and LBW, the study concluded that there was a trend toward women who experienced PTD and pPROM to have more severe periodontitis than mothers of full-term normal birth weight infants.[19] The proposed association was based on the hormonal changes associated with pregnancy that are significantly associated with other immune system cells that respond to chronic inflammation. Additionally, in 1998 Dasanayake *et al*. conducted a case-control study of 110 women and concluded that the presence of healthy gingiva, determined a lower risk for LBW infants.[16]

Current findings strengthen this theory that hormonal changes, primarily concerning estrogen and progesterone, are fully initiated around the 2nd month of pregnancy.[37, 66, 71] This could potentially be attributable to the 60-75% of pregnant women who begin to display clinical signs of gingivitis, making this the most common oral disease during pregnancy.[37, 90] The presence of gingivitis, even in an acute phase, increases a woman's risk for developing PD during and/or up to several months after pregnancy.[66, 71]

Since the landmark studies conducted in the 1990's, medical research has discovered that PTD is the leading cause of neonatal mortality in the US.[17] It is also responsible for 50% of the neurological handicaps occurring to children born too early.[72] Based on race and ethnicity, the rate of PTD is highest for black infants (18.1%) followed by Native Americans (13.8%), Hispanics (12%), Caucasian (11.5%) and Asians (10.5%). Maternal age is also significantly related to a mother's chances of experiencing a PTD. During a period between 2003-2005, PTD

was highest for women 40 years and older (16.6%), followed by women under age 20 who experienced 14.5% of all PTD.[17]

Adverse Pregnancy Outcomes: Impact on Healthcare

Not only has the management of an increasing number of PTD and LBW infants that survive in the NICU placed a significant monetary burden on the US health system, but PTD and LBW babies face multiple serious health issues all throughout life, which also demand monetary support. In 2005, the March of Dimes reported that the US spent about $26.2 billion dollars annually or $51,600 for every PTD infant, who represent about 10 % of all live North American births.[17] The average first-year medical costs, including both inpatient and outpatient care, were about 10 times greater for preterm infants ($32,325) than for term infants ($3,325). [17] It can also be reported that 60 **%** of all neonatal mortality is attributable to PTD which also accounts for nearly 50 **%** of all perinatal health care costs in the US. [38] Currently, the March of Dimes is leading a national campaign, whose goal is to meet the Healthy People 2010 objective of reducing the number of premature births to no more than 7.6 **%**, nearly half of the 12.1 **%** of premature births in 2002.[73]

Currently, the March of Dimes is leading a national campaign, whose goal is to meet the Healthy People 2010 objective of reducing the number of premature births to no more than 7.6 **%**, nearly half of the 12.1 **%** of premature births in 2002.[73] With these statistics in mind, it is important to remember that a woman's pregnancy experience with PD influences her oral health status, and more importantly, it significantly increases the risk of compromising the health of her fetus.[74]

Role of MCP-1 and RANTES in Pregnancy Outcomes

Because inflammation is hallmark of several female reproductive processes and due to Monocyte Chemoattractant Protien-1 (MCP-1) and Regulated upon activation, T-cell expressed or secreted (RANTES) involvement with many other inflammatory processes, this makes these two chemokines an interesting target for further research in association to pregnancy outcomes.

MCP-1 is involved with several aspects of uterine function, primarily present during the pre-menstrual stages and shortly after implantation of the fetus on the placental wall.[59] Furthermore, the endometrium, epithelial tissue that forms the lining of the uterus, has been shown to produce MCP-1 and RANTES among other chemokines such as IL-8, and MIP-1α.[27] RANTES also plays an important role in recruiting t-lymphocytes, monocytes, basophils and eosinophils into the endometrium.[59]

MCP-1 is a significant chemoattractant for specific subsets of lymphocytes, such as monocytes and macrophages.[32, 42, 54, 75] As the best-characterized member of the CC family, MCP-1 is expressed by monocytes, endothelial cells, fibroblasts and T-cells, primarily on the basal layer of epithelial tissues. [43, 49, 54, 76] MCP-1 has been associated with many inflammatory conditions such as granulamatous disease rheumatoid arthritis, heart disease, bone trauma and asthma. [54, 77] MCP-1 is related to the stages of oral infection by means of its monocyte chemotactic ability, as it has been known to increase with increasing inflammation. [45, 53]

RANTES is a member of the CC family as well, located on chromosome #17 in humans.[1] RANTES is believed to be a chemotactic factor for monocytes, macrophages, eosinophils, basophils and t-lymphocytes explaining why it's activity is commonly studied in conjunction with MCP-1.[40, 42, 54, 76, 78-79] Although research has not determined if it is directly related to chronic PD, Gamonal *et al.* conducted two studies in 2000, reporting that RANTES, along with Interleukin-10 (IL-10) were the only two chemokines exclusively associated with periodontitis patients. [24, 42] A study conducted by Nelson *et al.* also reported that events occurring in relation to the RANTES receptors indicate that RANTES is involved with acute and chronic stages of PD.[80]

During the studies of RANTES and PD, although still controversial, it was discovered that RANTES is potentially a significant chemoattractant of Th1 cells. [49, 81] Because Th1 cells are the cell-mediated response, primarily intensifying the presence of the immune cells that

invade inflammatory lesions, this supports the evidence that RANTES could be related to PD progression as well. [32]

For decades, research has consistently reported that an abundance of macrophage and monocyte cells are present in active sites of PD. [51, 55, 82-85] Macrophages constitute about 5-30% of the infiltrating cells in inflamed periodontal lesions and are often the most abundant cell of the chronic oral infection. [30, 43] Because many cytokines and chemokines have been associated with the recruitment of these cells to sites of inflammation, this suggests that these mediators are significantly involved with the immune response to periodontitis.[55] In 1993, Hanazawa *et al.* suggested that MCP-1 plays a role in the migration of monocytes into the periodontal tissues by reported the presence of MCP-1 in periodontitis patients and it's absence in periodontally healthy patients.[43] However, this study did not distinguish if MCP-1 levels increased with the severity of PD or if they were only associated with the presence of inflammation. Gamonal *et al* in 2000 also reported MCP-1's chemotactic ability on macrophages, is possibly causing the increase in severity of PD, due to an increased number of macrophages found in the GCF of active sites of PD. [24] Furthermore, they conducted the 1st study to show a presence of RANTES levels in patients with periodontitis and absence of RANTES in healthy patients. [42]

Research has shown several normal physiologic functions of female reproduction are initiated and regulated by inflammatory processes. [59] However, just as the literature concerning MCP-1, RANTES and PD is conflicting, the literature concerning these chemokines and pregnancy outcomes is inconsistent as well. In 1999, Athyde *et al.* concluded that women with preterm labor who also delivered preterm had higher amniotic fluid concentrations of RANTES than those who delivered term.[25] Microbial invasion of the amniotic cavity was also associated with a significant increase in median amniotic fluid RANTES in both preterm and term labor.[25] Additionally in 2003 Jacobsson *et al.* concluded that MCP-1 levels were higher in women experiencing preterm labor in association with inflammation than in non-laboring women,

suggesting that MCP-1 is only elevated in the presence of inflammation, pPROM and /or the microbial invasion of the amniotic cavity.[86]

In contrast to these findings, a cross-sectional study conducted by Esplin *et al.* in 2005 reported that amniotic levels of MCP-1 are increased in relation to all pathways of delivery, regardless of the presence or absence of inflammation. [23] Also in 2005, Tornblom *el al*. reported that MCP-1 levels were significantly increased during non-infected labor as compared with non-laboring women, again suggesting that MCP-1 is elevated in response to the gestational event and not necessarily the inflammation.[87] Finally, Ramhorst *el al.* concluded in 2007 that failure of RANTES levels to increase might be associated with increased risk for miscarriage while an epidemiological study, also conducted in 2007 found that elevated concentrations of RANTES, among other chemokines, were associated with higher risk of miscarriage.[88-89]

The vital question research asks today is whether or not cytokines and chemokines are responsible for any of the progressive pathogenesis, associated with inflammatory diseases.[51] Because of the current limitations in the literature concerning the association between PD, MCP-1, RANTES and pregnancy, the purpose of this secondary analysis was to compare the GCF levels of MCP-1 and RANTES in peri-partum women with periodontitis to healthy controls and correlate these levels with clinical parameters. In conjunction with evidence-based proof about the oral systemic link, studying these two chemokines prefaces a movement to accelerate the enhancement of oral and general health for all women and their children.

METHODS AND MATERIALS

This is secondary analysis of a cross sectional study conducted at Duke University Medical Center (DUMC) in collaboration with the University of North Carolina at Chapel Hill School of Dentistry (UNC). This study was approved by the DUMC and the UNC Institutional Review Boards. Eighty participants were stratified post hoc based on periodontal disease status. Healthy periodontal status was defined as $\leq$ 3mm periodontal probing depths and moderate to severe periodontal disease as $\geq$ 15 with $\geq$4mm periodontal probing depths. Exclusion criteria included non-English speaking women, minors without a guardian, gestation greater than 26 weeks, multiple gestation (twins or greater), chronic hypertension, diabetes, heart murmur or heart valve disease, history of Phen-fen use without documentation of a clear echocardiogram, any medical condition requiring antibiotic prophylaxis for dental treatment and HIV/AIDS. Demographic, health behavior, medical and pregnancy history data were obtained by patient questionnaire and were reviewed by a physician at the first prenatal visit. Calibrated dental examiners performed the oral health examination with direct data entry by a trained recorder.

An oral cancer screening was performed prior to the oral exam and if suspicious areas or lesions were noted the participant was referred to an oral pathologist. Missing teeth and implants were identified and were removed as data entry points. Sites for collecting plaque and gingival crevicular fluid (GCF) were computer generated. GCF samples were collected using filter paper prior to plaque sampling or probing measurements and were taken from the mesio-facial and distal-facial sites of the two most posterior teeth in each quadrant, excluding third molars. A perio-paper strip was placed in the gingival crevice and left until it was visually dampened. Fluid volume was determined using a calibrated Periotron 8000 (Oraflow Inc.). Fluid readings that fell out of the range of 30-180 were repeated and recorded. Once the fluid volume was determined it

was recorded in the direct data entry system. GCF strips were rolled securely in aluminum foil and placed into a pre-labeled cryovial and snap frozen in liquid nitrogen. All GCF samples were analyzed using the Luminex for MCP-1 and RANTES. The final component of the oral exam measured probing depth, cemento-enamel junction and bleeding upon probing. Gingival pocket depth was measured using a UNC-15 periodontal probe at six sites per tooth and rounded down to the next lower whole millimeter. Gingival recession and clinical attachment loss were measured and recorded. Bleeding upon probing was evaluated as presence or absence. Measurements and assessments were recorded in the direct data entry program. Descriptive statistics were used to calculate the demographic and oral health tables. Chi-square and *t*-tests were used to determine statistical significance at the level of 0.05. Unconditional logistic multivariate models were used to derive Odds Ratios and 95% Confidence Intervals.

RESULTS

The demographic characteristics are shown in tables 1 and 2. The levels of MCP-1 and RANTES are dichotomized at the 75th percentile because there is no specification about what value determines high as opposed to low, levels of these mediators.

Seventeen percent of cases were Caucasian compared to eighty-two percent of the controls ($p<0$ .0001). When comparing the levels of MCP-1 there were 19 Caucasian women and 41 African American women with low levels of MCP-1, with 65.5% of all Caucasian women and 80.4% of all AA having low levels MCP1. There were 10 Caucasian women and 10 AA women who had high MCP-1, with 34.5% of all Caucasian women and 19.6% of AA women having high MCP1. The difference between the rate of high MCP-1 amongst Caucasians and AA was not significant ($p=0.14$). However, there is a trend toward Caucasian women having higher levels of MCP-1.

There was a statistical difference ($p=0.008$) in the median/mean age of women and the levels of MCP-1 found in their crevicular fluid. The median/mean age of women with low levels of MCP-1 were 25 years old. However, for the women with high levels of MCP-1 the mean/median age was 30 years old. This suggests a trend towards increased levels of MCP-1 with age. This was not consistent with RANTES. In fact, there was no statistical difference in the levels of RANTES ($p=0.38$) based on age.

No statistically significant differences were found between cases and controls in mean GCF levels of MCP-1 (1.87(2.33), 1.95(2.47) $p=0.88$) and RANTES (1.81 (5.30), 1.97(5.78) $p=0.90$). The study also found that MCP-1 and RANTES were lower in cases than controls but this trend was not statistically significant. African American women were more likely to have

moderate/severe periodontal disease (Cases 17% Caucasian, controls 82% Caucasian ($p<0.0001$)).

A t-test analysis of the mean extent of BOP by high/low MCP-1 and RANTES showed a negative trend toward MCP-1, RANTES and BOP. The mean BOP for high MCP-1 ($\geq$10%) is lower (Mean BOP 24.9%) than for low MCP-1 (<10%) (Mean BOP 34.3) but it is not significant. For RANTES, there was no difference between high BOP ($\geq$10%) and low BOP (<10%). There were 26 women (32.5%) with low BOP, defined as <10% of all sites. There were 54 (67.5%) women with high BOP, defined as $\geq$10% of all sites. There were 35 (43.75%) of women with low BOP defined as <15% of all sites. There were 45 (56.25%) of women with high BOP, defined as $\geq$15% of all sites.

Adjustments made for moderate/severe PD and race showed no significance to elevated levels of MCP-1 (adj. odds ratio of 0.84 and 95% confidence interval of 0.44 to1.62) and RANTES (adj. odds ratio of 0.60 and 95% confidence interval of 0.32 to1.11). These values suggest that there is no relationship between elevated levels of MCP-1 and RANTES to moderate/severe PD and race. However, age is significantly related to elevated levels of MCP-1 (adj. Odds ratio of 1.11 and 95% confidence interval of 1.01 to 1.22). Preeclampsia, PPROM and smoking were not significantly related to elevated levels of MCP-1 or RANTES.

DISCUSSION

This secondary analysis evaluated the GCF levels of MCP-1 and RANTES of peri-partum women with periodontitis as compared with healthy controls. There were no statically significant increases of MCP-1 and RANTES found in the women with periodontitis. One limitation for this study was the definition of periodontal disease did not include bleeding upon probing. The definition used was consistent with other papers from the same data set. [90] However, bleeding on probing is an accurate indicator of active periodontitis and it would have been useful to further stratify participants.

Another limitation is that only English speaking women were enrolled. This was due to the limitation of the institution in providing translation services for research. However, according to the Institute of Medicine, maternal ethnicity is a significant risk factor for periodontal diseases and preterm delivery {{82 Institute of Medicine 2006;}}. It would have been beneficial for this study to include a variety of ethnic women, who speak a variety of languages, in order to better generalize the results.

To elicit further explanations on how MCP-1 and RANTES work with other bacteria it would have been beneficial to assay many species of bacteria; including *P. gingivalis*. This information may be helpful in explaining how MCP-1 does or does not contributes to periodontitis.

In order for normal labor to occur, an increase in inflammatory mediators is imperative. It is unknown whether MCP-1 or RANTES are directly involved with delivery. Future studies should consider the activity of inflammatory mediators that are directly associated with delivery, and compare those mediators to their involvement with periodontitis. This would help investigators

distinguish if these mediators are elevated due to delivery or the inflammation associated with periodontitis.

Although this study was unable to detect any increase in the levels of MCP-1 and RANTES in the GCF associated with periodontitis as compared with healthy controls, such data will be critical is helping to understand the complex processes associated with periodontitis and pregnancy outcomes.

CONCLUSION

This secondary analysis did not find any statistically significant differences between the GCF levels of MCP-1 and RANTES in peri-partum women with periodontitis as compared with healthy controls. Dental hygienists should continue to educate their pregnant patients about the risks associated with periodontal diseases during pregnancy. Further research is needed to determine the role of chemokines during periodontitis and gestational events.

TABLE 1

Demographics by High and Low GCF MCP-1 levels
Dichotomized at 75th percentile

	Low (Q1-Q3)	**High (Q4)**	**p-value**
Caucasian	19 (65.5%)	10 (34.5%)	
African American	41 (80.4%)	10 (19.6%)	0.14
Maternal Age Mean (StdDev)	25.6 (6.18)	30.1 (7.03)	**0.008***
Periodontal Healthy	27 (71.1%)	11 (29.0%)	
Mod/Severe	33 (78.6%)	9 (21.4%)	0.44
Bleeding on Probing Mean (StdDev)	34.3 (30.2)	24.9 (24.7)	0.22
Preeclampsia (Yes)	6 (54.6%)	5 (45.5%)	
No	54 (78.3%)	15 (21.7%)	0.09
PPROM (Yes)	16 (76.2%)	5 (23.8%)	
No	44 (74.6%)	15 (25.4%)	0.88

TABLE 2

Demographics by High and Low GCF RANTES levels
Dichotomized at the 75th percentile

	Low (Q1-Q3)	High (Q4)	p-value
Caucasian	23 (79.3%)	6 (20.7%)	
African American	36 (70.6%)	15 (29.4%)	0.39
Maternal Age Mean (StdDev)	27.1 (6.53)	25.6 (7.05)	0.38
Periodontal Healthy	26 (68.4%)	12 (31.6%)	
Mod/Severe	33 (78.6%)	9 (21.4%)	0.30
Bleeding on Probing Mean (StdDev)	31.9 (28.9)	31.9 (30.2)	0.99
Preeclampsia (Yes)	8 (72.7%)	3 (27.3%)	
No	51 (73.9%)	18 (26.1%)	0.93
PPROM (Yes)	14 (66.7%)	7 (33.3%)	
No	45 (76.3%)	14 (23.7%)	0.39

TABLE 3
Adjusted Logistic Model for Elevated MCP-1

	Odds Ratio (Confidence Interval)
Mod/Severe Periodontal Disease	0.84 (0.44-1.62)
Age (Years)	**1.11 (1.01-1.22)***
Caucasian	1.00 (0.24-4.18)

TABLE 4
Adjusted Logistic Model for Elevated RANTES

	Odds Ratio (Confidence Interval)
Mod/Severe Periodontal Disease	0.60 (0.32-1.11)
Age (Years)	0.99 (0.91-1.09)
Caucasian	0.37 (0.09-1.60)

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